A Method to Determine Cytochrome P4501A Activity in Wildlife Microsomes

S.F. Trudeau and F.J. Maisonneuve

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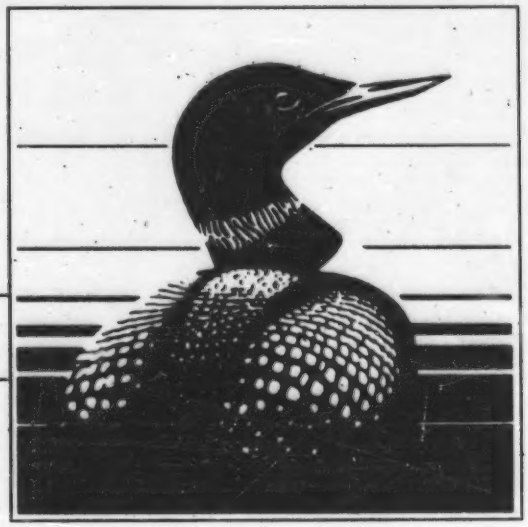

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A METHOD TO DETERMINE CYTOCHROME P4501A ACTIVITY IN WILDLIFE MICROSOMES

S.F. Trudeau and F.J. Maisonneuve

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PREFACE

The Biomarker Laboratory (Laboratory Services Section) of the National Wildlife Research Centre (NWRC) has been providing biochemical analyses (biomarkers) in wildlife tissues in support of its National Wildlife Toxicology Program since the early 1990s.

Induction of cytochrome P4501A is a sensitive biomarker of exposure to environmental contaminants such as PAHs and HAHs. Measurement of the cytochrome P450-associated enzyme, 7-ethoxyresorufin-*O*-deethysase (EROD), is the most common method to evaluate the activity of the P4501A system. This report contains a detailed description of a fluorescent spectrophotometric method (**MET-BMK-EROD-01**), to measure the product of the EROD reaction (resorufin) and total protein simultaneously, using a plate reader. It also contains, in Appendix A, the details for preparation of microsomes by gel filtration and, in Appendix B, a procedure adapted for smaller liver samples (< 200 mg). Preparation of microsomes by ultracentrifugation is given in Appendix C.

This report has been prepared to provide a citable reference for our clients and collaborators. Standard Operating Procedures (SOPs) specific to our organization are cited throughout this document and SOPs that are not included in this report can be obtained from the authors, upon request.

The names of manufacturers, suppliers and trade names are included only to document the exact assay conditions adopted by the NWRC. Other equivalent products, instruments or reagents from other sources may also give satisfactory results.

PRÉFACE

Méthode pour l'analyse de l'activité du cytochrome P4501A dans des microsomes d'animaux sauvages.

Depuis le début des années 1990, le Laboratoire de dépistage biologique de la Section des services de laboratoire du Centre national de la recherche faunique (CNRF) fourni, dans le cadre de son Programme national de surveillance des effets des produits toxiques sur les espèces sauvages, un service d'analyses biochimiques ou biomarqueurs.

L'induction du cytochrome P4501A est un biomarqueur sensible à l'exposition aux contaminants environnementaux tels que les HAP (hydrocarbures aromatiques polycycliques) et les HAH (hydrocarbures aromatiques halogénés). La mesure de l'activité de l'enzyme associée au système des cytochromes P450, la 7-éthoxyrésorufine-*O*-déethylase (EROD), est la méthode la plus courante pour évaluer l'activité du système P4501A. Le présent document contient une description détaillée d'une méthode fluorométrique (**MET-BMK-EROD-01**) appliquée au lecteur de plaques, qui permet de mesurer simultanément le produit de la réaction de EROD (résorufine) et les protéines dans les microsomes hépatiques. On y retrouve, à l'annexe A, les détails pour la préparation des microsomes par filtration sur gel et, à l'annexe B, une procédure adaptée pour les foies de plus petite taille (<200 mg). La préparation de microsomes par ultracentrifugation est présentée à l'annexe C.

Ce rapport a été écrit dans le but spécifique de fournir une référence pouvant être citée par nos clients et collaborateurs. Tout au long du document on renvoi le lecteur à des modes opératoires normalisés (« *SOPs* ») qui sont spécifiques à notre organisation. Les modes opératoires qui ne sont pas inclus dans ce rapport peuvent être obtenus en communiquant directement avec les auteurs.

Le nom des manufacturiers, fournisseurs et nom de commerce des produits sont inclus uniquement dans le but de documenter les conditions d'analyse précises utilisées par le CNRF. Des produits, instruments ou réactifs équivalents provenant d'autres sources peuvent aussi donner des résultats satisfaisants.

ABBREVIATIONS

7-ER	7-ethoxyresorufin
BSA	bovine serum albumin
EM	emission wavelength (nm)
EROD	7-ethoxyresorufin-*O*-deethylase
EX	excitation wavelength (nm)
HAHs	planar halogenated aromatic hydrocarbons
MFOs	mixed function oxygenases
NADPH	nicotinamide adenine dinucleotide phosphate
NIST	National Institute of Standards and Technology
PAHs	polycyclic aromatic hydrocarbons
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
ε	molar absorptivity coefficient

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EROD ACTIVITY MEASUREMENT IN WILDLIFE MICROSOMES

1. SCOPE AND FIELD OF APPLICATION

This is a spectrofluorometric method for the measurement of the cytochrome P4501A-associated enzyme, 7-ethoxyresorufin-*O*-deethylase (EROD) in microsomes. It is an adaptation of the method published by Kennedy and Jones (1994).

2. REFERENCES

2.1. Kennedy, S.W. and Jones, S.P. (1994) Simultaneous measurement of cytochrome P4501A catalytic activity and total protein concentration with a fluorescence plate reader. *Anal. Biochem.*, 222, 217-223.

2.2. Burke, M.D., Prough, R.A. and Mayer, R.T. (1977) Characteristics of a microsomal cytochrome P-448-mediated reaction. Ethoxyresorufin *O*-de-ethylation. *Drug Metab. Dispos.*, 5, 1-8.

2.3. Pohl, R.J. and Fouts, J.R. (1980) A rapid method for assaying the metabolism of 7-ethoxyresorufin by microsomal subcellular fractions. *Anal. Biochem.*, 107, 150-155.

2.4. Hewlett Packard (1986) HP 8452A diode array spectrophotometer handbook. Publication No: 08452-90001 and software handbook.

2.5. Millipore, CytoFluor 2300/2350 fluorescence measurement system user's guide.

3. PRINCIPLES AND DEFINITIONS

Mixed function oxygenases (also called mixed function oxidases, MFOs or polysubstrate multifunction oxygenases) are a family of membrane bound enzymes which add oxygen to a wide range of compounds, including endogenous substances, rendering them more water soluble and thus more readily excreted. This group of enzymes, which are major components of the biological defense that protects living organisms from toxic compounds, is induced by a variety of environmental contaminants. The induction of cytochrome P4501A (CYP1A) is a well known biochemical response observed in vertebrates upon exposure to polyaromatic hydrocarbons (PAHs) and planar halogenated aromatic hydrocarbons (HAHs), such

as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Measurement of CYP1A catalytic activity using the 7-ethoxyresorufin-*O*-deethylase assay is a biomarker commonly used to determine exposure to, and possible effects of, PAHs and HAHs in wild birds, mammals and amphibians.

This method is based on the formation of resorufin from 7-ethoxyresorufin, a reaction which is catalyzed by CYP1A in the presence of NADPH. The reaction is stopped by precipitating the microsomal proteins with acetonitrile containing fluorescamine, which reacts with the primary amino groups of the protein to produce a fluorescent compound which is measured in a fluorescence plate reader (EX400/EM460). Protein quantification is done against a BSA standard curve. In the same reaction mixture, resorufin is measured (EX530/EM590) and quantified against a resorufin standard curve. Reactions are carried out in a 48-well plate with BSA and resorufin standards in each plate.

4. Reagents, Solutions, Materials and Standards

SAFETY PRECAUTIONS

⇒ General safety rules and waste disposal procedures that apply to the Biomarker Laboratory must be followed. (Ref. Safety Manual)

⇒ Adequate protective equipment must be used: lab coat, gloves, mask (when homogenizing tissues), cryogloves and face shield (when retrieving samples stored in liquid nitrogen).

⇒ Material Safety Data Sheets (MSDS) for the products used must be read.

4.1. Reagents

4.1.1. Sodium phosphate dibasic, Na_2HPO_4, M.W. 141.96, Fisher S-374

4.1.2. Sodium phosphate monobasic, $NaH_2PO_4 \cdot H_2O$, M.W. 137.99, Fisher S-369

4.1.3. Resorufin sodium salt, M.W. 235.2, Sigma R-3257

4.1.4. 7-ethoxyresorufin, M.W. 241.2, Sigma E-3763

4.1.5. Nicotinamide adenine dinucleotide phosphate (NADPH), M.W. 833.4, Sigma N-6505

4.1.6. Methanol (MeOH), HPLC grade

4.1.7. Bovine serum albumin (BSA), purity 98-99%, Sigma A-7030

4.1.8. Fluorescamine, M.W. 278.3, Sigma F-9015

4.1.9. Acetonitrile, HPLC grade

4.1.10. De-ionized water from the Milli-RO/Milli-Q system (Millipore)

4.2. Solutions

4.2.1. *Sodium phosphate dibasic 0.05 M* - Dissolve 7.1 g Na_2HPO_4 in ca 250 mL of purified water in a 1 L volumetric flask then complete to volume.

4.2.2. *Sodium phosphate monobasic 0.05 M* - Dissolve 3.45 g $NaH_2PO_4 \cdot H_2O$ in ca 250 mL of purified water in a 500 mL volumetric flask then complete to volume.

4.2.3. *Sodium phosphate buffer 0.05 M, pH 8.0* - Bring solutions 4.2.1 and 4.2.2 to 37°C. In a 2 L beaker containing 1 L of solution 4.2.1, add solution 4.2.2 until pH reaches 8.0 (ca 100 mL of solution 4.2.2 is required).

4.2.4. *7-Ethoxyresorufin (7-ER) 1 mM in MeOH* - ***stock solution***

4.2.4.1. Dissolve a few crystals of 7-ER in ca 1 mL MeOH using the sonicator.

4.2.4.2. In a 5 mL graduated tube, add 60 µL of solution 4.2.4.1 and dilute to 3.0 mL with MeOH.

4.2.4.3. Measure the absorbance at 470 nm using the HP8452 spectrophotometer.

4.2.4.4. Calculate the solution concentration using $\varepsilon_{470\ nm}$ = 16/mM.cm (Pohl, 1980) and multiply this value by 50 to obtain the concentration of the stock solution (before its dilution). ***Note:*** if the stock solution is not 1000 ± 100 µM, either add more 7-ER or more MeOH.

4.2.4.5. Transfer the stock solution into an amber bottle and keep on ice. - ***Shelf life*****:** prepare fresh daily

4.2.5. *7-Ethoxyresorufin 9.4 µM* - ***working solution***

Dilute the stock solution (4.2.4.5) in buffer. Due to the instability of this solution, prepare at the last minute and use immediately. The final concentration in the reaction mixture (total vol.: 235 µL) will be 2 µM.

Note: MeOH is an enzyme inhibitor, consequently always adjusting the concentration of the stock solution (in MeOH) to 1 mM will ensure that the amount of MeOH in the reaction mixture is the same.

4.2.6. *Fluorescamine 2.16 mM in acetonitrile* - Dissolve 60 mg in 100 mL of acetonitrile. Keep on ice during the assay. ***Shelf life***: stable for 1 week at 4°C.

4.2.7. *NADPH 2.4 mM in phosphate buffer* - Dissolve 20 mg in 10 mL of buffer (NADPH is **very unstable**, prepare at the last minute - step 7.3.16). The final concentration in the reaction mixture (total vol.: 235 µL) will be 0.5 mM.

4.3. Standards

4.3.1. *BSA 2 mg/mL in phosphate buffer* - Add 200 mg to a 100 mL volumetric flask containing ca 20 mL of phosphate buffer and **slowly stir** (to minimize foam formation) with a magnetic bar until completely dissolved. Slowly add buffer to complete to volume and mix by gently inverting the flask to avoid foaming. Filter a few mL of the solution through a 0.22 µm MSI filter. Add 1 mL of the filtered aliquot to 2 mL of buffer in a quartz cuvette. Determine the concentration by measuring the absorbance at 280 nm (Abs_{280nm}: 0.66 for 1 mg/mL). Keep the filtered solution on ice while preparing the plate.

4.3.2. *Resorufin 150 µM in MeOH - **stock solution*** - Weigh ca 4 mg of resorufin in 100 mL of MeOH and sonicate for 5 min. The concentration of the stock solution is determined by using 200 µL of the stock solution diluted to 3.0 mL with buffer. Absorbance is measured at 572 nm (ε_{572nm} = 73.2/mM.cm). ***Shelf life:*** a few weeks at 4°C.

4.3.3. *Resorufin 3 µM - **working solution*** - Add 200 µL of the stock solution to a 10 mL volumetric flask and complete to volume with buffer. ***Note:*** prepare just before use (ref. step 7.3.9).

4.4. Quality Control Sample

Microsomes prepared in-house and preserved in LN_2 are used to monitor the day-to-day variation of the method. Select a sample with similar matrix and similar activity as the analytical sample and include one on each plate.

4.5. Blanks

Sample blank wells contain all the components (i.e., buffer, microsomes, ER, acetonitrile/fluorescamine) with the exception of the NADPH. Standard blank wells contain buffer, ER, NADPH and acetonitrile/fluorescamine (ref. **Table 1**).

5. AUXILIARY EQUIPMENT

5.1. Glassware and Labware

- **5.1.1.** Rainin M8 pipet (or multichannel pipet of equivalent precision)
- **5.1.2.** Eppendorf pipets, 10-100 µL and 100-1000 µL (or pipets of equivalent precision)
- **5.1.3.** Eppendorf Repeater pipetter with Combitip (or pipet of equivalent precision)
- **5.1.4.** SMI pipet, 10-15-25 µL with glass capillaries
- **5.1.5.** B&D multifit glass syringe, 5 cc with MSI filters Cameo 25NS, 0.22 µm (Fisher DDN02025S0)
- **5.1.6.** Disposable cuvettes, 1 cm lightpath (Sarstedt 67.738)
- **5.1.7.** Quartz cuvettes, 1 cm lightpath
- **5.1.8.** Volumetric flasks, 10, 25, 100 and 1000 mL
- **5.1.9.** Plates with lids, 48 wells, Falcon 3078 (VWR 62 406 195)
- **5.1.10.** Beakers, 250 mL and 1 L
- **5.1.11.** Graduated tubes, 5 mL
- **5.1.12.** Glass vial, 20 mL

5.2. Equipment

- **5.2.1.** pH meter (PerpHect meter - Model 350)
- **5.2.2.** Analytical balance (Sartorius RC210P) and top-loading balance (Mettler PM4000)
- **5.2.3.** Sonicator (Branson 1200)
- **5.2.4.** Vortex mixer
- **5.2.5.** Magnetic stirrer and magnetic bar
- **5.2.6.** Timer
- **5.2.7.** Dry block heater (VWR 13259-009)

5.3. Instrumentation

5.3.1. Fluorescence multiwell plate reader (Cytofluor Model 2350, Millipore Ltd.), equipped with a tungsten-halogen lamp and a Hamamatsu R929 red-sensitive photomultiplier. The instrument is controlled with Millipore 2300 version 3b1 operating software.

5.3.2. Spectrophotometer (Hewlett-Packard photodiode-array, HP8452) controlled with HP89530A MS™-DOS UV/VIS (version 1) operating software.

6. Specimen or Sample Handling Requirements

Tissues are collected and preserved as recommended in the document "Protocol for Field Collection and Storage of Wild Birds for Biomarker Studies" (S. Trudeau, Biomarker Laboratory, NWRC, 1992). Tissues and microsomes to be used in this assay **must be stored in LN_2** and methods for collecting, processing and storing liver samples and microsomal fractions must be the same for samples pertaining to a particular study to allow comparability of results.

7. Procedure

7.1. Sample Preparation

Microsomes are prepared by gel filtration (ref. SOP-BMK-PROC-08 and 12 in appendix) or by ultracentrifugation (ref. SOP-BMK-PROC-14 in appendix). Twenty microsome samples are thawed on ice at one time (thawing time: ca 1 h). ***Note*:** minimum sample size recommended: 200 µL of microsomal fraction.

7.2. Instrument Operating Parameters

7.2.1. Computer file path: C:\CYTO (to access the software from Windows, double click on the Cyto icon)

7.2.2. Filter sets #1 (to measure resorufin): EX: C 530/25, EM: C 590/35, sensitivity 3 or 4

7.2.3. Filter sets #2 (to measure fluorescamine proteins): EX: E 400/35, EM: F 460/40, sensitivity 3.

7.3. Analysis

7.3.1. Turn on the CytoFluor, spectrophotometer and dry block heater (adjusted to 37°C for avian and mammalian species).

7.3.2. Remove a maximum of 20 tubes of microsomes from LN_2 and allow them to thaw on ice.

7.3.3. Weigh the NADPH into a 20 mL glass vial and keep the powder on ice.

7.3.4. Prepare stock solutions of 7-ER, BSA and resorufin as described in 4.2.4, 4.3.1 and 4.3.2 respectively and determine their concentrations.

7.3.5. Fill out the "EROD Worksheet" (FORM-BMK-12) describing the plate configuration. ***Note***: four plates can be prepared at one time.

7.3.6. With a permanent marker, identify the plates and the lids.

7.3.7. With the multichannel pipet, add buffer to the wells (details in **Table 1**).

7.3.8. With the multichannel pipet, add BSA solution to the wells identified "Standards" (details in **Table 1** - one standard blank and 5 different concentrations, each in triplicate).

7.3.9. Dilute the resorufin stock solution in buffer (200 µL in 10 mL).

7.3.10. With the multichannel pipet, add the resorufin solution to the wells identified "Standards" (details in **Table 1** - one standard blank and 5 different concentrations, each in triplicate).

7.3.11. Vortex the microsomes. With an SMI pipet add 10, 15 or 25 µL of microsomes to the corresponding wells (each sample is done in triplicate plus one sample blank). ***Note***: volume of microsomes is selected to fall within the limits of the standard curves (see 10.7). Sample blank wells also receive microsomes.

7.3.12. Dilute the 7-ER stock solution in buffer (ref. section 4.2.5).

7.3.13. Add 50 µL of solution 7.3.13 in each well using the Eppendorf Repeater pipetter.

7.3.14. Secure the plates in the dry block heater and pre-incubate at 37°C for 5 min.

7.3.15. While pre-incubating, dissolve the pre-weighed NADPH (20 mg) in 10 mL of buffer.

7.3.16. In a timely fashion, start the reaction by adding NADPH to the sample wells with the Eppendorf Repeater pipetter (**do not add NADPH to sample blanks**). Then proceed with the standard wells, with the same time interval. Proceed the same way with plates 2, 3 and 4. ***Note***: if 4 plates were prepared, record on each lid the starting time of the reaction.

7.3.17. Incubate the plates for 10 min at 37°C (otherwise record the exact incubation time).

7.3.18. Stop the reaction by adding 100 µL of cold acetonitrile (containing fluorescamine - section 4.2.6) at the same time interval as the NADPH was added. ***Note***: acetonitrile/fluorescamine is also added to the sample blanks and each plate is removed from the block heater before adding acetonitrile to the next plate.

7.3.19. Immediately scan the plates, starting with plate 1 as described below (refer to the manufacturer's operation manual for more detailed instructions).

7.4. Configuring a Scan

7.4.1. Pull down the SYSTEM menu and choose PLATE SCAN.

7.4.2. Pull down the FILE menu and click on NEW.

7.4.3. Enter filename and comments.

7.4.4. Define the plate type (i.e., Falcon 48)

7.4.5. Ensure that the parameters correspond to the ones described in section 7.2.

7.5. Scanning and Saving File

7.5.1. Place the plate transport mechanism in the load position.

7.5.2. Secure the plate no.1 onto the plate transport mechanism. *Note:* well A1 should be at the top right.

7.5.3. From the SCAN menu, select SCAN.

7.5.4. Once the scan is completed, from the DATA menu, click on PRINT.

7.5.5. In the FILE menu, click on EXPORT.CSV.

7.5.6. Select the appropriate directory, and assign a filename. The extension "CSV" will automatically be given.

7.5.7. Verify if the sensitivity chosen was appropriate and proceed with plate no. 2. *Note*: if the sensitivity gives "99999" values, it indicates that the fluorescence exceeds the instrument's limit and the plate should be read at a lower sensitivity.

Table 1 - Typical position and volumes (in µL) of standards, QC reference material and samples in the 48-well plate

	Well #	Buffer[a]	BSA	Resorufin	Microsomes[a]	ER	NADPH	Acetonitrile/ Fluorescamine
Standards								
	1 A-C	•	•	•	•	•	•	•
	1 D-F	•	•	•	•	•	•	•
	2 A-C	135	•	•	•	50	50	100
	2 D-F	120	10	5	•	50	50	100
	3 A-C	105	20	10	•	50	50	100
	3 D-F	75	40	20	•	50	50	100
	4 A-C	50	60	25	•	50	50	100
	4 D-F	15	80	40	•	50	50	100
Samples								
QC sample blank	A 5	170	•	•	15	50	•	100
QC sample	A 6-8	120	•	•	15	50	50	100
Sample 1 blank	B 5	170	•	•	15	50	•	100
Sample 1	B 6-8	120	•	•	15	50	50	100
Sample 2 blank	C 5	170	•	•	15	50	•	100
Sample 2	C 6-8	120	•	•	15	50	50	100
Sample 3 blank	D 5	170	•	•	15	50	•	100
Sample 3	D 6-8	120	•	•	15	50	50	100
Sample 4 blank	E 5	170	•	•	15	50	•	100
Sample 4	E 6-8	120	•	•	15	50	50	100
Sample 5 blank	F 5	170	•	•	15	50	•	100
Sample 5	F 6-8	120	•	•	15	50	50	100

[a] Amount of buffer and microsomes may vary but total volume in each well must be 335 µL

8. EXPRESSION OF RESULTS

The rate of enzyme activity is reported in pmole of resorufin formed per minute per mg of total protein.

8.1. Calculations

8.1.1. Fluorescence data are imported into Quattro-PRO ver. 5.0 for Windows (Borland) for curve fitting.

8.1.2. Open the template file "TEMPLATE.ROD".

8.1.3. From the cell "1A" in the "fluor" page (tab name), import the "*.CSV" file obtained in section 7.3.20.

8.1.4. In the "protein" page (tab name), enter the filename, the plate no., the BSA solution concentration and the sample identification. Ensure that the BSA volumes used are identical to the ones appearing on the template (BSA column).

8.1.5. In the "resorufin" page (tab name), enter the resorufin working solution concentration. Ensure that the resorufin volumes used are identical to the ones appearing on the template (resorufin column). If incubation time is not 10 min make the appropriate changes (EROD activity column).

8.1.6. In the "macro" page (tab name), go to cell "4B" and execute the macro. Calculations (detailed in 8.2 and 8.3 below) will be done automatically and the results will be printed. Ref. section 9.0 for representative documents.

8.2. Total Protein Determination

Total proteins are derived from a BSA standard curve. The BSA curve is non-linear and corresponds to a quadratic equation. The quadratic polynomial curve fit equation is:

$$F = a+bx+cx^2$$

where: F= BSA standards fluorescence (fluorescence units)
x = BSA standards concentration ($\mu g\ mL^{-1}$)
a, b and c are coefficients determined by the non-linear regression of the BSA standard curve.

Once the coefficients a, b and c are determined they are used for the following mathematical transformation:

$$z = \{b^2-[4(a-\text{sample fluorescence})]c\}^{0.5}$$
$$y = (-b+z)/2c$$

where: y = protein concentration in the sample well ($\mu g\ mL^{-1}$)

$$\text{Total protein (mg)} = y\ (\mu g\ mL^{-1}) * 0.335\ mL/1000\ (\mu g\ mg^{-1})$$

where 0.335 mL = total vol. in the well after addition of acetonitrile/fluorescamine

8.3. Resorufin Determination

The resorufin formed is derived from a resorufin standard curve using the following equation:

$$y = mx + b$$

where: y = sample fluorescence in fluorescence units (after blank correction)
m = slope of the resorufin standard curve (fluorescence units nM^{-1})
x = resorufin concentration in the sample wells in nM
b = Y-intercept (0 in this case)

The resorufin concentration in the well in nM is then converted to pmol. The specific activity is then calculated:

$$\frac{[\text{resorufin}]\ nM * 335 \times 10^{-6}\ L * 1000\ pmol\ nmol^{-1}}{min * \text{total protein (mg)}}$$

where: 335×10^{-6} = total volume
min = reaction time

9. REPRESENTATIVE DOCUMENTS

Figure 1 - Cytofluor 2300 print-out

CYTOFLUOR 2300 SYSTEM REPORT

Lab: 95c79c10 Wed Jun 28 15:24:09 1995
File: 280695-007 28JUN01A
Assay: erod
Plate: Falcon 48

Set	EX Filter	EM Filter	Sensitivity
1	530/25	590/35	3
2	400/35	460/40	3

Comments:

Scan No.: 1 of 1 Page No.: 1

		1	2	3	4	5	6	7	8
A	Set 1	19	39	377	866	48	556	567	557
	Set 2	63	72	350	1820	1980	1942	1909	1926
B	Set 1	19	38	378	852	45	1228	1214	1204
	Set 2	67	74	847	1780	1888	1888	1888	1904
C	Set 1	19	39	381	857	49	1242	1194	1228
	Set 2	59	60	866	1785	1926	1980	1920	2003
D	Set 1	19	221	680	1288	46	611	596	604
	Set 2	61	461	1366	2048	1841	1893	1862	1904
E	Set 1	19	221	688	1314	47	367	356	368
	Set 2	60	451	1370	2072	1841	1810	1830	1867
F	Set 1	19	216	686	1317	48	285	288	283
	Set 2	62	455	1390	2131	1716	1711	1716	1716

Figure 2 - Protein Calculations

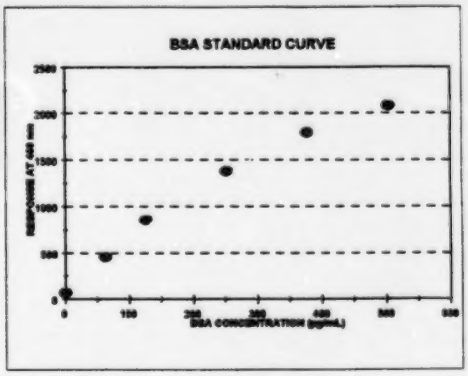
DATE: 280695
FILNAME: E28JUN01.WB1
PLATE NO. JUN01A.CSV
SENSITIVITY 3

BSA STANDARD CURVE

BSA concentration = 2.11 mg/mL

BSA (µg/mL)	(BSA)^2	FLUORESCENCE avg (n=3)	std	%var
0	0	89	8	11.03
63	3967	456	5	1.10
126	15868	854	10	1.20
252	63474	1375	13	0.93
378	142816	1795	22	1.21
504	253896	2084	43	2.05

Regression Output:		
Constant		79.32871
Std Err of Y Est		28.67883
R Squared		0.999195
No. of Observations		6
Degrees of Freedom		3
X Coefficient(s)	5.44816714	-0.004942
Std Err of Coef.	0.25027892	0.000482



SAMPLES

	SAMPLE NO.	FLUORESCENCE replicate	response	Z [1]	Y [2]	TOTAL PROTEIN IN WELL mg (from curve fit values)
QA	93CARHG6-10	1	1942	2.1814	431.6958	0.1446
		2	1909	2.3261	417.0535	0.1397
		3	1926	2.2527	424.4790	0.1422
	91KHG1	1	1888	2.4137	408.1924	0.1367
		2	1888	2.4137	408.1924	0.1367
		3	1904	2.3473	414.9138	0.1390
	91PRHG6	1	1980	2.0018	449.8637	0.1507
		2	1920	2.2789	421.8310	0.1413
		3	2003	1.8848	461.6991	0.1547
	91PRHG8	1	1893	2.3931	410.2728	0.1374
		2	1862	2.5179	397.6482	0.1332
		3	1904	2.3473	414.9138	0.1390
	91KHG3	1	1810	2.7144	377.7715	0.1266
		2	1830	2.6405	385.2413	0.1291
		3	1887	2.4982	399.6417	0.1339
	91PRHG10	1	1711	3.0536	343.4442	0.1151
		2	1716	3.0374	345.0860	0.1156
		3	1716	3.0374	345.0860	0.1156

QUADRATIC POLYNOMIAL CURVE FIT: $F = a + b * x + c * x^2$

Fit F to y (BSA standards fluorescence)

The coefficients a, b & c are determined by the non-linear regression

a=	79.33
b=	6.45
c=	-0.00494

MATH TRANSFORMATION:
[1] $Z = [(b)^2-[4*(a\text{-samples fluorescence})]*c)]^{0.5}$
[2] $Y = (-b+Z)/(2*c)$

TOTAL PROTEINS [3] (Y (µg ml-1) * 0.335 ml) / 1000 µg mg-1

Figure 3 - Resorufin Calculations

DATE: 280695
FILNAME: E28JUN01.WB1
PLATE NO. 28JUN01A.CSV
SENSITIVITY 3

RESORUFIN STANDARD CURVE

Resorufin concentration = 3.1 *µM*

RESORUFIN (nM)	FLUORESCENCE avg (n=3)	-blank	std	% var
0	39	0	1	1.49
46	219	181	3	1.32
93	379	340	2	0.55
185	685	646	4	0.61
231	858	820	7	0.83
370	1308	1268	16	1.22

Regression Output:	
Constant	0
Std Err of Y Est	18.28486
R Squared	0.99816
No. of Observations	5
Degrees of Freedom	4
X Coefficient(s)	3.474834
Std Err of Coef.	0.03768

SAMPLES

	SAMPLE NO.	FLUORESCENCE blank	replicate	response	-blank	EROD ACTIVITY [1]	PROTEINS (mg)	EROD SPECIFIC ACTIVITY [2]	AVG n=3	STD	% VAR.
QA	93CARHG6-10	48	1	556	508	4.90	0.1446	33.87	34.73	0.99	2.86
			2	567	519	5.00	0.1397	35.82			
			3	557	509	4.91	0.1422	34.51			
	91KHG1	46	1	1228	1183	11.41	0.1367	83.41	82.07	1.64	1.87
			2	1214	1169	11.27	0.1367	82.42			
			3	1204	1159	11.17	0.1390	80.39			
	91PRHG6	49	1	1242	1193	11.50	0.1507	76.32	75.98	2.33	3.07
			2	1194	1145	11.04	0.1413	78.12			
			3	1228	1179	11.37	0.1547	73.49			
	91PRHG8	48	1	611	565	5.45	0.1374	39.63	39.24	0.64	1.64
			2	598	547	5.27	0.1332	39.59			
			3	604	556	5.35	0.1390	38.50			
	91KHG3	47	1	367	320	3.09	0.1266	24.38	23.53	0.74	3.14
			2	356	309	2.98	0.1291	23.08			
			3	368	321	3.09	0.1339	23.12			
	91PRHG10	48	1	285	237	2.28	0.1151	19.86	19.82	0.21	1.06
			2	288	240	2.31	0.1156	20.02			
			3	283	236	2.27	0.1156	19.60			

[1] pmol RESORUFIN/min = FLUORESCENCE UNITS * A
Where A = (335 exp-6 (L) / [SLOPE (f.u.* nmol-1* L) x 10 min]) * (1000 pmol nmol-1)
= 9.84E-03

[2] pmol RESORUFIN/min/mg PROTEINS = [1] / PROTEINS

10. QUALITY CONTROL

10.1. Blanks

One blank well is prepared for each sample (ref. Section 4.5) and its fluorescence is subtracted from the sample fluorescence.

Blank correction is also made with the standards using the average of triplicate measurements (ref. Section 4.5).

10.2. Standards

The concentration of the resorufin and BSA is verified spectrophotometrically. Standard curves done with five different concentrations (in triplicates) are included on each plate.

10.3. Quality Control Sample

Microsomes prepared in house, divided into small aliquots and stored in LN_2 are used. A quality control sample is included on each plate. Control charting and corrective actions are done as described in SOP-BMK-DOC-03, which also details non-conforming criteria and corrective actions to be taken.

10.4. Temperature

Avian and mammalian microsomes are assayed at 37°C. Optimal temperature must be determined for other animal species. The temperature of the dry block heater is verified against a NIST calibrated thermometer each year.

10.5. Microsome Volumes

Microsomes are added to the well with a SMI pipet and the protein concentration in each well is determined to compensate for any variation in the delivery volume.

10.6. Sample Accountability

Possible sources of error inherent in the collection, transport, and reception of specimen for analysis and any initial processing steps are recorded (details are included in SOP-BMK-DOC-04). Non-conforming situations are reported on form FORM-TP-06 (and corrective actions are applied if necessary).

10.7. Linearity

The amounts of microsomes are adjusted to have fluorescence values which fall within the standard curves. Normally 10 to 25 µL of microsomes is used but fluorescence response depends on enzyme activity and protein concentration of microsomes. If fluorescence response is outside the limits of the standard curves, the assay is repeated with either a different sample volume or a different standard concentration range. In addition, the incubation time chosen must allow the reaction to proceed linearly (ref. Kennedy and Jones, 1994).

10.8. Precision

Analyses are done in triplicate and repeated if results differ by more than 10% at normal levels and 15% at low levels.

10.9. Randomization of Samples

To avoid any biased results, samples are analyzed at random, not taking into account the sample collection date or site.

10.10. Standard Operating Procedures

Other related SOPs used in this assay include:

- *SOP-BMK-PROC-07* for pipet calibration
- *SOP-BMK-PROC-01* for reagent solutions
- *SOP-BMK-PROC-03* for glassware cleaning
- *SOP-BMK-PROC-09* for temperature verification
- *SOP-BMK-PROC-10* for balance calibration
- *SOP-BMK-MAIN-01* for spectrophotometer calibration
- *SOP-BMK-MAIN-03* for pH meter verification

Calibration protocols for pipets, thermometers, balances, spectrophotometer and pH meter are detailed in these procedures as well as the calibration frequency required.

11. Critical Control Points

- Microsome samples must be kept on ice until the preparation of the plate.

- The 7-ethoxyresorufin working solution (in the buffer) and the NADPH solution must be prepared at the last minute.

- Avian and mammalian microsomes are incubated at 37°C. Optimal temperature must be determined for other animal species.

- The incubation time (i.e., the time interval between the addition of NADPH and the addition of the acetonitrile/fluorescamine) must be well monitored and allow the reaction to proceed linearly (ref. Kennedy and Jones, 1994).

APPENDIX A

SOP-BMK-PROC-08

PREPARATION OF LIVER MICROSOMES BY GEL FILTRATION

1. SCOPE AND FIELD OF APPLICATION

This is a procedure for the isolation of hepatic microsomes by gel filtration, adapted from Pyykko, 1983. Microsomes are used to measure the activity of 7-ethoxyresorufin-*O*-deethylase (MET-BMK-EROD-01) and other detoxifying enzymes. A minimum of 1.25 g of tissue (wet weight) is required for this procedure. For smaller sample sizes, refer to SOP-BMK-PROC-12 in Appendix B of this report.

2. PRINCIPLES

In this method, microsomes are prepared by homogenizing minced liver with a buffer, centrifuging the homogenate and letting the supernatant flow by gravity down a column packed with Sepharose CL-2B which has previously been washed and equilibrated with the buffer. Particles of different molecular size travel through the porous gel at different rates permitting the isolation of the microsomal enzymes.

3. DEFINITIONS

Microsomes are small membrane-enclosed vesicles derived from the endoplasmic reticulum of fractionated cells. They contain several enzymes involved in xenobiotic metabolism.

4. REAGENTS AND SOLUTIONS

SAFETY PRECAUTIONS

⇒ General safety rules and waste disposal procedures that apply to the Biomarker Laboratory must be followed. (Ref. Safety Manual)

⇒ Adequate protective equipment must be used: lab coat, gloves, mask (when homogenizing tissues), cryogloves and face shield (when retrieving samples stored in liquid nitrogen).

⇒ Material Safety Data Sheets (MSDS) for the products used must be read.

4.1. Reagents

4.1.1. Potassium chloride, KCl, M.W. 74.56, Fisher P-217

4.1.2. Potassium phosphate monobasic, KH_2PO_4, M.W. 136.09, Fisher P-285

4.1.3. Sodium phosphate dibasic, Na_2HPO_4, M.W. 141.96, Fisher S-374

4.1.4. Acetone, HPLC grade

4.1.5. Sepharose CL-2B, Pharmacia 17-0140-01

4.1.6. Purified water from the Milli-RO/Milli-Q system.

4.2. Solutions

4.2.1. *Potassium chloride 0.15 M* - Dissolve 22.4 g KCl in ca 500 mL of purified water in a 2 L volumetric flask then complete to volume. Prepare 2 x 2 L.

4.2.2. *Sodium phosphate 0.1 M* - Dissolve 28.4 g Na_2HPO_4 in ca 500 mL of purified water in a 2 L volumetric flask then complete to volume. Prepare 2 x 2 L.

4.2.3. *Potassium phosphate 0.1 M* - Dissolve 13.6 g KH_2PO_4 in ca 250 mL of purified water in a 1 L volumetric flask then complete to volume.

4.2.4. *Phosphate buffer 0.1 M, pH 7.4* - Add 200 mL potassium phosphate solution to 800 mL of sodium phosphate solution. Adjust pH, if necessary, with H_3PO_4 or NaOH. Prepare 5 L.

5. AUXILIARY EQUIPMENT

5.1. Glassware and Labware

5.1.1. Filtering flask with tubulation, 1 L (with rubber stopper)

5.1.2. Büchner funnel, 13 cm diam. (with rubber adapter)

5.1.3. Filter paper, Whatman no.1, 9 cm diam. (Fisher 09-805D)

5.1.4. Econo-Columns (10 mm x 30 cm) with stopcock and end caps (Bio-Rad 737-1031)

5.1.5. Econo-Columns rack (Bio-Rad 731-7000)

5.1.6. Beakers 25 mL and 1 L

5.1.7. Volumetric flasks, 1 L and 2 L

5.1.8. Ice bucket (to keep buffer and samples cold)

5.1.9. Disposable transfer pipets and Pasteur pipets

5.1.10. Weighing dish

5.1.11. Petri dishes

5.1.12. Scissors (angular blade), scalpels and spatula

5.1.13. Centrifuge tube Oakridge, 10, 30 or 50 mL

5.1.14. Graduated cylinder, 10 mL and 25, 50 or 100 mL

5.1.15. Plastic funnels, 60 mm top diam.

5.1.16. Gauze bandage

5.1.17. Disposable centrifuge tubes with caps

5.1.18. Plastic beaker (filled with ice to keep homogenate cold during homogenization)

5.1.19. Parafilm

5.1.20. Cryovials (1.2 mL), vial racks, CryoCane holders and CryoCane sleeves

5.1.21. Disposable test tubes (13x100 mm)

5.1.22. Dispensette with 500 mL bottle (to dispense buffer)

5.1.23. Pipets (Pipetman P-1000 and Biohit 5000 - or pipets of equivalent precision)

5.1.24. Plastic vials to collect eluate (ca 25 mL capacity)

5.1.25. Liquid nitrogen container

5.2. Equipment

5.2.1. Magnetic stirrer and magnetic bar

5.2.2. Vacuum pump (Edwards - VWR Scientific no. EVA 362-02-980)

5.2.3. pH meter (PerpHect meter - Model 350)

5.2.4. Top-loading balance, precision 0.01 g (Mettler PM4000)

5.2.5. Tissue homogenizer (Heidolph RZR-2000) with Wheaton Potter-Elvehjem tissue grinders of appropriate size

5.2.6. Refrigerator (door model, Cryo-Fridge, American Scientific Products)

5.3. Instrumentation

5.3.1. Centrifuge (Beckman JA-21) with various rotors

6. HANDLING OF SPECIMEN

Liver samples must be stored in liquid nitrogen immediately after collection (within ca 5 min of sacrificing). Detailed procedure is described in "Protocols for field collection and storage of wild bird specimens for biomarker studies" (S. Trudeau, Biomarker Laboratory, NWRC, 1992). **All operations involved in microsome preparation must be done at 0-4°C. Cold reagent solutions and glassware must be used. After preparation, microsomes must be stored immediately in liquid nitrogen until the assay.**

7. PROCEDURE

7.1. Gel Preparation

7.1.1. Turn on the refrigeration unit on the vacuum pump about one hour prior to gel preparation. Follow manufacturer's instructions for refrigeration unit and pump operation.

7.1.2. Add ca 300 mL of gel (Sepharose CL-2B) to a 1 L beaker. *Note*: enough to prepare 10 columns.

7.1.3. Add an equal amount of acetone and mix well with a spatula.

7.1.4. Assemble Büchner funnel apparatus and attach to the vacuum pump. Filter the gel solution through a filter paper Whatman #1.

7.1.5. Transfer the gel to the 1 L beaker and add, for the second time, an equal amount of acetone. Mix well.

7.1.6. Filter and repeat the operation once more (i.e., acetone is added and filtered 3 times in total).

7.1.7. Transfer the gel to the 1 L beaker and rinse with an equal volume of 0.15 M KCl solution. Mix well and filter.

7.1.8. Repeat step 7.1.7 approximately 10 times, until no acetone odour is detected.

7.1.9. Resuspend the gel in ca 800 mL phosphate buffer, allow to settle 1 h in the refrigerator and then decant. ***Note:*** in the mean time, cryovials can be identified and put in freezer as described in step 7.4.1.

7.1.10. Repeat step 7.1.9 two more times and then decant excess buffer to give a solution of gelatinous consistency.

7.1.11. Transfer the gel into a filtering flask attached to the vacuum pump, top with a rubber stopper (upside down) and degas the solution (about 10-20 min) using a magnetic stirrer to stir the solution.

7.1.12. Degas the buffer needed for subsequent steps the same way.

7.2. Column Preparation

7.2.1. Install stopcocks on columns, in the closed position. ***Note:*** usually 10 columns are prepared at one time.

7.2.2. Secure columns on rack and let them cool in the refrigerator.

7.2.3. Add a few mL of buffer to each column and let it drain into vials placed below each column (20 mL plastic vials can be used). Let the buffer drain until the meniscus reaches the fritted disk (inert support) then close the stopcock. ***Note:*** this is done to saturate the column fritted disk with the buffer and remove air space.

7.2.4. With the Dispensette, add 16 mL of buffer and mark on the column (with a permanent marker) the volume it occupies (bottom of meniscus). This line will indicate the amount of gel to be added to the column in order to have a ratio of gel to sample 4:1. ***Note:*** in this method 4 mL of sample will be added.

7.2.5. Drain 75% of the buffer and then adjust the stopcock to give a drop by drop flow.

7.2.6. When almost all the buffer is drained, carefully pour the slurry of gel along the side of the column, up to a few cm over the marked line. The

gel must be added in a single operation. Allow the adsorbent to settle by gravity.

7.2.7. After the bed has formed, allow the excess liquid on the top of the column to enter the bed surface until ca 1 cm remains above the bed surface. **At no time during the column packing or sample application should the adsorbent be allowed to go "dry".**

7.2.8. If more gel needs to be added to the column after the packing is completed, add a few mL of buffer and with a Pasteur pipet gently resuspend the top 2-3 cm of the bed by stirring and pour additional slurry.

7.2.9. If gel needs to be removed, resuspend with a few mL of buffer and remove excess material with a Pasteur pipet.

7.2.10. Inspect the column. The absorbent bed must be free of bubbles, cracks or spaces between the gel and the walls of the column.

7.2.11. Fill the column completely with buffer and allow the buffer to flow just to the top of the gel.

7.2.12. Repeat step 7.2.11 then close the outlet and add a few mL of buffer. Cap the columns.

7.2.13. Discard the eluates.

7.3. Preparation of Liver Homogenate

***Note*: Buffer, glassware, apparatus, etc. must all be chilled before being used. Keep them in the refrigerator at 4°C.**

7.3.1. Thaw liver samples on ice. Large liver samples (e.g., 10 g) may be thawed overnight on ice in a refrigerator (4°C). Samples to be prepared in the afternoon can be removed from the liquid nitrogen storage container in the morning and thawed on ice in a refrigerator (4°C). ***Note:*** ca 10 samples per day can be prepared with this method.

7.3.2. Transfer the liver to a weighing dish and record its weight. At least 1.25 g of tissue is required.

7.3.3. Put the liver in a cold Petri dish (kept on ice) and rinse a few times with small amounts of cold phosphate buffer solution. Remove rinsing buffer with a transfer pipet and discard.

7.3.4. Mince the liver into small pieces with cold scissors or scalpels, keeping the Petri dish on ice at all times. ***Note:*** at this stage, pre-refrigerate the centrifuge and rotor at 4°C (ref. step 7.3.13).

7.3.5. Quantitatively transfer the minced liver into a Wheaton tissue grinder of appropriate size.

7.3.6. If necessary, rinse the Petri dish with a small amount of cold buffer and add the rinses to the tissue grinder. ***Note:*** use only a few mL of buffer as the amount added should not exceed the total volume defined in section 7.3.10 below.

7.3.7. Put crushed ice into a plastic beaker and insert the homogenizing tube in.

7.3.8. Secure a previously cooled Potter-Elvehjem pestle in place and rinse with cold buffer.

7.3.9. Homogenize the liver with the homogenizer set at maximum speed until the pestle reaches the bottom of the tube, then homogenize further with the speed adjusted to 400 RPM, using 6 up and down strokes (keeping the tube in the ice at all time).

7.3.10. Quantitatively transfer the homogenate to a previously cooled graduated cylinder of appropriate size and make up to 5 volumes with chilled buffer, using initial liver lobe weight to do the calculation (e.g., 3 g liver + 12 mL buffer for a total volume of 15 mL). Record volume of buffer added.

7.3.11. Cover the cylinder with Parafilm or stopper and mix well by inversion.

7.3.12. Transfer the homogenate to an Oakridge centrifuge tube of appropriate size (do not fill tube completely, e.g., add a maximum of 20 mL of homogenate in 30 mL tube). ***Note:*** Keep unused homogenate portion on ice until microsome preparation is completed then discard.

7.3.13. Equilibrate the tubes in rotor and spin at 12 000 g (10 500 RPM for rotor JA-18 and 12 000 RPM for rotor JA-21 on the Beckman

centrifuge) for 15 min at 4°C - ref. Operator's manual for details. ***Note:*** this step removes cell debris, nuclei and mitochondria.

7.3.14. Cover plastic funnels with gauze cloth (4 or 5 layers).

7.3.15. Decant the supernatant through gauze to remove chilled fats. Collect the postmitochondrial supernatant in disposable centrifuge tubes, applying pressure on the gauze with a spatula to collect most of the material. Proceed with gel filtration.

7.4. Isolation of Microsomes by Gel Filtration

7.4.1. Identify 1.2 mL cryovials (10 vials per sample); include project no., date, sample no., and MICR (microsomes). Place in freezer.

7.4.2. Identify cryocane sleeves and prepare a liquid nitrogen container.

7.4.3. Equilibrate column with 2 x 8 mL of buffer. Gently add buffer along the column wall and avoid compacting or resuspending the gel. Five samples (i.e., 5 columns) can be prepared at the same time.

7.4.4. Load 4 mL of supernatant (ref. step 7.3.15) on column and discard eluate.

7.4.5. Rinse the column walls with 1 mL of buffer and discard eluate.

7.4.6. Place 10 mL graduated cylinder under each column.

7.4.7. Load 10 mL elution buffer and collect 7 mL of out flowing eluate as microsomal fraction. Haemoglobin and other soluble proteins remain in the column.

7.4.8. Transfer the well-mixed microsomal fractions to previously identified cryovials. ***Note*:** add ca 700 µL per tube, keeping the cryovials on ice.

7.4.9. Secure cryovials in aluminum canes and cover with an identified cryocane sleeve. **Immediately store in liquid nitrogen**.

7.5. Column Clean up

7.5.1. Wash column with at least 20 mL buffer. If there is fat on the top of column, remove with a pipet, resuspend the gel, and readjust gel volume. Cap the columns. ***Note***: columns can be used up to 4 times before gel must be discarded and repacked with freshly washed gel.

8. CRITICAL CONTROL POINTS AND DISCUSSION

- Buffer, glassware, apparatus, etc. must all be chilled before being used.
- At no time during the column packing or sample application should the adsorbent be allowed to go dry.
- Microsomes should be immediately stored in liquid nitrogen after their preparation.
- Samples pertaining to a particular study must be processed and stored the same way.
- The gel filtration method is useful when no ultracentrifuge is available. Another advantage is the excellent separation of microsomes from solute proteins and especially from haemoglobin contamination (Tangen -1973; Capevilla -1975; cited in Pyykko -1983).
- Microsomes prepared by gel filtration are more dilute than microsomes prepared by ultracentrifugation.

9. REFERENCES

9.1. Pyykko, K. (1983) Characterization and stability of rat liver microsomes isolated by a rapid gel filtration method. *Acta Pharmacol. et Toxicol.*, 52 , 39-46.

9.2. Beckman (1985) Model J2-21M/E Centrifuge -Instruction Manual.

APPENDIX B

SOP-BMK-PROC-12

PREPARATION OF LIVER MICROSOMES BY GEL FILTRATION WITH SMALL AMOUNT OF LIVER

1. SCOPE AND FIELD OF APPLICATION

This is a method for the isolation of hepatic microsomes by gel filtration for small sizes of samples. It is an adaptation of Pyykko, 1983. Microsomes are used to measure the activity of 7-ethoxyresorufin-*O*-deethylase (MET-BMK-EROD-01) and other detoxifying enzymes. A minimum of 200 mg of tissue (wet weight) is required.

2. PRINCIPLE

In this method, microsomes are prepared by homogenizing minced liver with a buffer, centrifuging the homogenate and letting the supernatant flow by gravity down a column packed with Sepharose CL-2B which has previously been washed and equilibrated with the buffer. Particles of different molecular size travel down the column at different rates permitting the isolation of the microsomal enzymes.

3. DEFINITIONS

Microsomes are small membrane-enclosed vesicles derived from the endoplasmic reticulum of fractionated cells. They contain several enzymes involved in xenobiotic metabolism.

4. REAGENTS AND SOLUTIONS

SAFETY PRECAUTIONS

⇒ General safety rules and waste disposal procedures that apply to the Biomarker Laboratory must be followed. (Ref. Safety Manual)

⇒ Adequate protective equipment must be used: lab coat, gloves, mask (when homogenizing tissues), cryogloves and face shield (when retrieving samples stored in liquid nitrogen).

⇒ Material Safety Data Sheets (MSDS) for the products used must be read.

4.1. Reagents

4.1.1. Potassium chloride, KCl, M.W. 74.56, Fisher P-217

4.1.2. Potassium phosphate monobasic, KH_2PO_4, M.W. 136.09, Fisher P-285

4.1.3. Sodium phosphate dibasic, Na_2HPO_4, M.W. 141.96, Fisher S-374

4.1.4. Acetone, HPLC grade

4.1.5. Sepharose CL-2B, Pharmacia 17-0140-01

4.1.6. Purified water from the Milli-RO/Milli-Q system.

4.2. Solutions

4.2.1. *Potassium chloride 0.15 M* - Dissolve 22.4 g KCl in ca 500 mL of purified water in a 2 L volumetric flask then complete to volume. Prepare 2 x 2 L.

4.2.2. *Sodium phosphate 0.1 M* - Dissolve 28.4 g Na_2HPO_4 in ca 500 mL of purified water in a 2 L volumetric flask then complete to volume. Prepare 2 x 2 L.

4.2.3. *Potassium phosphate 0.1 M* - Dissolve 13.6 g KH_2PO_4 in ca 250 mL of purified water in a 1 L volumetric flask then complete to volume.

4.2.4. *Phosphate buffer 0.1 M, pH 7.4* - Add 200 mL potassium phosphate solution to 800 mL of sodium phosphate solution. Adjust pH, if necessary, with H_3PO_4 or NaOH. Prepare 5 L.

5. AUXILIARY EQUIPMENT

5.1. Glassware and Labware

5.1.1. Filtering flask with tubulation, 1 L (with rubber stopper)

5.1.2. Büchner funnel, 13 cm diam. (with rubber adapter)

5.1.3. Filter paper, Whatman no.1, 9 cm diam. (Fisher 09-805D)

5.1.4. Econo-Columns (7 mm x 20 cm) with stopcock and end caps (Bio-Rad 737-0721)

5.1.5. Econo-Columns rack (Bio-Rad 731-7000)

5.1.6. Beakers 25 mL and 1 L

5.1.7. Volumetric flasks, 1 L and 2 L

5.1.8. Ice bucket (to keep buffer and samples cold)

5.1.9. Disposable transfer pipets and Pasteur pipets

5.1.10. Aluminum weighing dish (Fisher 8-732)

5.1.11. Petri dishes

5.1.12. Scissors (angular blade) or scalpels and spatula

5.1.13. Eppendorf microcentrifuge tube 1.8 mL

5.1.14. Plastic beaker (filled with ice to keep homogenate cold during homogenization)

5.1.15. Cryovials (1.2 mL), vial racks, CryoCane holders and CryoCane sleeves

5.1.16. Dispensette, 10 mL with 500 mL bottle (to dispense buffer)

5.1.17. Pipets (Pipetman P-1000)

5.1.18. Plastic vials to collect eluate (ca 25 mL capacity)

5.1.19. Liquid nitrogen container

5.2. Equipment

5.2.1. Magnetic stirrer and magnetic bar

5.2.2. Vacuum pump (Edwards - VWR Scientific no. EVA 362-02-980)

5.2.3. pH meter (PerpHect meter - Model 350)

5.2.4. Top-loading balance, precision 0.01 g (Mettler PM4000)

5.2.5. Tissue homogenizer (Heidolph RZR-2000) with Wheaton Potter-Elvehjem tissue grinders of appropriate size

5.2.6. Refrigerator (door model, Cryo-Fridge, American Scientific Products)

5.3. Instrumentation

5.3.1. Centrifuge (Beckman JA-21) with rotor JA18.1

6. HANDLING OF SPECIMEN

Liver samples must be stored in liquid nitrogen immediately after collection (within ca 5 min of sacrificing). Detailed procedure is described in "Protocols for field collection and storage of wild bird specimens for biomarker studies" (S. Trudeau, Biomarker Laboratory, NWRC, 1992). **All operations involved in microsome preparation must be done at 0-4°C. Cold reagent solutions and glassware must be used. After preparation, microsomes must be stored immediately in liquid nitrogen until the assay.**

7. PROCEDURE

7.1. Gel Preparation

7.1.1. Turn on the refrigeration unit on the vacuum pump about one hour prior to gel preparation. Follow manufacturer's instructions for refrigeration unit and pump operation.

7.1.2. Add ca 300 mL of gel (Sepharose CL-2B) to a 1 L beaker. ***Note*:** enough to prepare 10 columns.

7.1.3. Add an equal amount of acetone and mix well with a spatula.

7.1.4. Assemble Büchner funnel apparatus and attach to the vacuum pump. Filter the gel solution through a filter paper Whatman #1.

7.1.5. Transfer the gel to the 1 L beaker and add, for the second time, an equal amount of acetone. Mix well.

7.1.6. Filter and repeat the operation once more (i.e., acetone is added and filtered 3 times in total).

7.1.7. Transfer the gel to the 1 L beaker and rinse with an equal volume of 0.15 M KCl solution. Mix well and filter.

7.1.8. Repeat step 7.1.7 approximately 10 times, until no acetone odour is detected.

7.1.9. Resuspend the gel in ca 800 mL phosphate buffer, allow to settle 1 h in the refrigerator and then decant. ***Note:*** in the mean time, cryovials can be identified and put in freezer as described in step 7.4.1.

7.1.10. Repeat step 7.1.9 two more times and then decant excess buffer to give a solution of gelatinous consistency.

7.1.11. Transfer the gel into a filtering flask attached to the vacuum pump, top with a rubber stopper (upside down) and degas the solution (about 10-20 min) using a magnetic stirrer to stir the solution.

7.1.12. Degas the buffer needed for subsequent steps the same way.

7.2. Column Preparation

7.2.1. Install stopcock on columns (7 mm x 20 cm), in the closed position. ***Note:*** usually 10 columns are prepared at one time.

7.2.2. Secure columns on rack and let them cool in the refrigerator.

7.2.3. Add a few mL of buffer to each column and let it drain into vials placed below each column (20 mL plastic vials can be used). Let the buffer drain until the meniscus reaches the fritted disk (inert support) then close the stopcock. ***Note***: this is done to saturate the column fritted disk with the buffer and remove air space.

7.2.4. With the Dispensette, add 4 mL of buffer and mark on the column (with a permanent marker) the volume it occupies (bottom of meniscus). This line will indicate the amount of gel to be added to the column in order to have a ratio of gel to sample 4:1. ***Note***: in this method 1 mL of sample will be added.

7.2.5. Drain approximately 75% of the buffer and then adjust the stopcock to give a drop by drop flow.

7.2.6. When almost all the buffer is drained, carefully pour the gel along the side of the column with a Pasteur pipette, up to a few cm over the marked line. Allow the adsorbent to settle by gravity.

7.2.7. After the bed has formed, allow the excess liquid on the top of the column to enter the bed surface until ca 1 cm remains above the bed

surface. **At no time during the column packing or sample application should the adsorbent be allowed to go "dry".**

7.2.8. If more gel needs to be added to the column after the packing is completed, add a few mL of buffer and with a Pasteur pipet gently resuspend the top 1-2 cm of the bed by stirring and pour additional slurry.

7.2.9. If gel needs to be removed, resuspend with a few mL of buffer and remove excess material with a Pasteur pipet.

7.2.10. Inspect the column. The absorbent bed must be free of bubbles, cracks or spaces between the gel and the walls of the column.

7.2.11. Fill the column completely with buffer and allow the buffer to flow just to the top of the gel.

7.2.12. Repeat step 7.2.11 then close the outlet and add a few mL of buffer. Cap the columns.

7.2.13. Discard the eluates.

7.3. Preparation of Liver Homogenate

Note: **Buffer, glassware, apparatus etc. must all be chilled before being used. Keep them in the refrigerator at 4°C.**

7.3.1. Thaw liver samples on ice (30 min to 1 h). *Note:* ca 10 samples per day can be prepared with this method.

7.3.2. Transfer the liver to an aluminum weighing dish and record its weight. At least 200 mg of tissue is required.

7.3.3. Put the liver in a cold Petri dish (kept on ice) and rinse a few times with small amounts of cold phosphate buffer solution. Remove rinsing buffer with a transfer pipet and discard.

7.3.4. Mince the liver into small pieces with cold scissors or scalpels, keeping the Petri dish on ice at all time. *Note:* at this stage, pre-refrigerate the centrifuge and rotor at 4°C (ref. step 7.3.13).

7.3.5. Quantitatively transfer the minced liver into a 2 mL Potter-Elvehjelm tissue grinder.

7.3.6. Add 800 µL of cold buffer with P-1000 pipette.

7.3.7. Place homogenizing tube in a plastic beaker containing crushed ice and homogenize manually with 10 up and down strokes (keeping the tube in the ice at all times).

7.3.8. Quantitatively transfer the homogenate to a previously cooled 1.8 mL Eppendorf microcentrifuge tube.

7.3.9. Equilibrate the tubes in rotor and centrifuge at 9 000 g (9 000 RPM for rotor JA-18.1) for 15 min at 4°C. ***Note***: this step removes cell debris, nuclei and mitochondria.

7.3.10. Decant the supernatant (S-9 fraction) into a clean Eppendorf microcentrifuge tube. Proceed with gel filtration.

7.4. Isolation of Microsomes by Gel Filtration

7.4.1. Identify 1.2 mL cryovials (2 vials per sample); include project no., date, sample no., and MICR (for microsomes). Place in freezer.

7.4.2. Identify CryoCane sleeves and prepare a liquid nitrogen container.

7.4.3. Equilibrate column with 4 mL of buffer. Gently add buffer along the column wall and avoid compacting or resuspending the gel. Five samples (i.e., 5 columns) can be prepared at the same time.

7.4.4. Transfer all the supernatant obtained in step 7.3.10 (ca 1 mL) on column and discard flowing eluate.

7.4.5. Rinse the column walls with 1 mL of buffer and discard eluate.

7.4.6. Place 1.25 mL graduated tube under each column.

7.4.7. Load 5 mL elution buffer and collect 1.25 mL of out flowing eluate as microsomal fraction. Haemoglobin and other soluble proteins remain in the column.

7.4.8. Transfer the well-mixed microsomal fractions to previously identified cryovials. ***Note*:** add ca 600 µL per tube, keeping the cryovials on ice.

7.4.9. Secure cryovials in aluminum canes and cover with an identified cryocane sleeve. **Immediately store in liquid nitrogen**.

7.5. Column Clean up

As described in Section 7.5 of SOP-BMK-PROC-08 but using at least 10 mL buffer instead of 20.

8. Critical Control Points and Discussion

- Buffer, glassware, apparatus, etc. must all be chilled before being used.
- At no time during the column packing or sample application should the adsorbent be allowed to go dry.
- Microsomes should be immediately stored in liquid nitrogen after their preparation.
- Samples pertaining to a particular study must be processed and stored the same way.
- The gel filtration method is useful when no ultracentrifuge is available. Another advantage is the excellent separation of microsomes from solute proteins and especially from haemoglobin contamination (Tangen -1973; Capevilla -1975; cited in Pyykko -1983).
- Microsomes prepared by gel filtration are more dilute than microsomes prepared by ultracentrifugation.

9. References

9.1. Pyykko, K. (1983) Characterization and stability of rat liver microsomes isolated by a rapid gel filtration method. *Acta Pharmacol. et Toxicol.*, 52 , 39-46.

9.2. Beckman (1985) Model J2-21M/E Centrifuge -Instruction Manual.

APPENDIX C

SOP-BMK-PROC-14

PREPARATION OF LIVER MICROSOMES BY ULTRACENTRIFUGATION

1. SCOPE AND FIELD OF APPLICATION

This is a method for the isolation of hepatic microsomes by ultracentrifugation. Microsomes are used to measure the activity of 7-ethoxyresorufin-*O*-deethylase (MET-BMK-EROD-01) and other detoxifying enzymes. Approximately 200-300 mg of tissue (wet weight) is required.

2. PRINCIPLE

In this method, a liver homogenate (suspension of cell organelles and tissue debris) is prepared by homogenization: by rotating the tightly fitted Teflon pestle in the glass tube, cells are disrupted by shearing forces. Differential centrifugation is then used to fractionate the liver homogenate into its various constituent organelles. The first centrifugation step at 9 000 g removes the cell debris, nuclei and mitochondria. The resulting supernatant (S-9 fraction) is then centrifuged at 100 000 g for 60 min and the microsomal fraction is obtained by re-suspending the pellet in a buffer.

3. DEFINITIONS

Microsomes are small membrane-enclosed vesicles derived from the endoplasmic reticulum of fractionated cells. They contain several enzymes involved in xenobiotic metabolism

4. REAGENTS AND SOLUTIONS

SAFETY PRECAUTIONS

⇒ General safety rules and waste disposal procedures that apply to the Biomarker Laboratory must be followed. (Ref. Safety Manual)

⇒ Adequate protective equipment must be used: lab coat, gloves, mask (when homogenizing tissues), cryogloves and face shield (when retrieving samples stored in liquid nitrogen).

⇒ Material Safety Data Sheets (MSDS) for the products used must be read.

4.1. Reagents

4.1.1. Potassium phosphate monobasic, KH_2PO_4, M.W. 136.09, Fisher P-285

4.1.2. Sodium phosphate dibasic, Na_2HPO_4, M.W. 141.96, Fisher S-374

4.1.3. Purified water from the Milli-RO/Milli-Q system.

4.2. Solutions

4.2.1. *Sodium phosphate 0.1 M* - Dissolve 14.2 g Na_2HPO_4 in ca 200 mL of purified water in a 1 L volumetric flask then complete to volume.

4.2.2. *Potassium phosphate 0.1 M* - Dissolve 3.4 g KH_2PO_4 in ca 100 mL of purified water in a 250 mL volumetric flask then complete to volume.

4.2.3. *Phosphate buffer 0.1 M, pH 7.4* - Add 200 mL potassium phosphate solution to 800 mL of sodium phosphate solution. Adjust pH, if necessary, with phosphoric acid (H_3PO_4) or sodium hydroxide (NaOH).

5. AUXILIARY EQUIPMENT

5.1. Glassware and Labware

5.1.1. Beakers, 1 L and 2 L

5.1.2. Volumetric flasks, 250 mL and 1 L

5.1.3. Ice bucket (to keep buffer and samples cold)

5.1.4. Disposable transfer pipets, 1 mL (Fisher 13-711-7)

5.1.5. Aluminum weighing dish (Fisher 8-732)

5.1.6. Pyrex Petri dishes

5.1.7. Scissors (angular blade) or scalpels and spatula

5.1.8. Wheaton Potter-Elvehjem tissue grinder (2 mL)

5.1.9. Cotton swab (Fisher 14-959-92B)

5.1.10. Eppendorf microcentrifuge tubes,1.8 mL (Fisher 05-664-22)

5.1.11. Beckman Polyallomor thick wall centrifuge tube, 3.2 mL (Beckman 362 333)

5.1.12. Plastic beaker (filled with ice to maintain homogenate cold during homogenization)

5.1.13. Cryovials 1.25 mL (Sarstedt 72-694-007) or 1.0 mL (Nunc 366656), vial racks, CryoCane holders, CryoCane sleeves

5.1.14. Pipets (Pipetman P-1000) or equivalent

5.1.15. Liquid nitrogen container

5.2. Equipment

5.2.1. Magnetic stirrer and magnetic bar

5.2.2. pH meter (PerpHect meter - Model 350)

5.2.3. Top-loading balance, precision 0.01 g

5.2.4. Tissue homogenizer (Heidolph RZR-2000)

5.3. Instrumentation

5.3.1. Centrifuge (Beckman JA-21) with appropriate rotor (JA-18.1 or JA-21)

5.3.2. Ultracentrifuge (Beckman Optima TL) with rotor (TLA 100.4)

6. HANDLING OF SPECIMEN

Liver samples must be stored in liquid nitrogen immediately after collection (within ca 5 min of sacrificing). Detailed procedure is described in "Protocols for field collection and storage of wild bird specimens for biomarker studies" (S. Trudeau, Biomarker Laboratory, NWRC, 1992). **All operations involved in microsome preparation must be done at 0-4°C. After preparation, microsomes must be stored immediately in liquid nitrogen until the assay.**

7. PROCEDURE

7.1. Preparation of Liver Homogenate

***Note*: Buffer, glassware, apparatus etc. must all be chilled before being used. Keep them in the refrigerator at 4°C.**

7.1.1. Thaw liver on ice. ***Note:*** between 16 to 24 samples per day can be prepared with this method.

7.1.2. Transfer the liver to a weighing dish and record its weight. At least 200 mg of tissue is required.

7.1.3. Put the liver in a cold Petri dish (kept on ice) and rinse a few times with small amounts of cold phosphate buffer solution. Remove rinsing buffer with a transfer pipet and discard.

7.1.4. Mince the liver into small pieces with cold scissors or scalpels keeping the Petri dish on ice at all time. ***Note:*** at this stage, pre-refrigerate the centrifuge and rotor at 4°C.

7.1.5. Quantitatively transfer the minced liver into a 2 mL Wheaton homogenizing tube.

7.1.6. Add 800 µL of cold buffer with P-1000 pipet.

7.1.7. Put crushed ice into a plastic beaker. While holding the homogenizing tube in ice at all times, homogenize the liver tissue with 10 up and down strokes using the Heidolph homogenizer (maximum speed).

7.1.8. Quantitatively transfer the homogenate into a previously cooled 1.8 ml Eppendorf microcentrifuge tube.

7.1.9. Equilibrate the tubes in the rotor and spin at 9 000 g (9 000 RPM for rotor JA-18.1 on the Beckman JA-21centrifuge) for 15 min. at 4°C - ref. Operator's manual for details.

7.1.10. Quantitatively decant the supernatant (S-9 fraction) to a cold 3.2 mL Beckman polyallomor thick wall centrifuge tube and proceed with ultracentrifugation.

7.2. Isolation of Microsomes by Ultracentrifugation

7.2.1. Identify 4 cryovials per sample; include project no., date, sample no., and MICR (for microsomes). Place in freezer.

7.2.2. Identify CryoCane sleeves and prepare a liquid nitrogen container.

7.2.3. Refrigerate the ultracentrifuge and rotor at 4°C.

7.2.4. Equilibrate the tubes in the rotor and centrifuge at 100 000 g (50 000 RPM) on the Beckman Optima TL ultracentrifuge for 60 min at 4°C. - ref. Operator's manual.

7.2.5. Decant the supernatant. ***Note*:** The supernatant (cytosol fraction) can be discarded or put aside for cytosolic enzymatic assays if desired. At this point, if there is any fat remaining on the side of the tube, remove it with a cotton swab. Avoid touching the pellet.

7.2.6. Insert the Wheaton Teflon directly in the Beckman polyallomor centrifuge tube and detach the gel pellet from the bottom of the tube by moving the tube around the pestle in small circular movements. ***Note:*** the Beckman polyallomor thick wall centrifuge tube's inside diameter is larger than the pestle's diameter.

7.2.7. While maintaining the pestle in motion in the tube, add 100 µl of cold buffer and re-suspend the "gel-like" pellet. Keep the tube in crushed ice at all times.

7.2.8. Still maintaining the pestle in motion in the tube, add 900 µl of cold buffer to the microsomal fraction. About 5-7 circular movements of the pestle in the bottom of the tube is needed to obtain an homogenous microsomal suspension.

7.2.9. Transfer approximately 250 µl of the well-mixed microsomal fraction to each previously identified cryovial (ref. 7.2.1). ***Note:*** put the cryovials on ice during this operation.

7.2.10. Secure cryovials in aluminum canes and slide in a well identified CryoCane sleeve. **Immediately store in liquid nitrogen**.

8. CRITICAL CONTROL POINTS

- Buffer, glassware, apparatus etc. must all be chilled before being used.
- Microsomes should be immediately stored in liquid nitrogen after their preparation.
- Samples pertaining to a particular study must be processed and stored the same way.

APPENDIX D

FORM-BMK-12

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EROD WORKSHEET

Analyst: **Date:**

Method:

Project no.:	**Notebook no.:**	**Plate no.:**
Project leader:	**Page:**	

- BSA concentration: _____ mg/ml
- Resorufin concentration (working solution): _____ µM
- 7-ethoxyresorufin concentration (in reaction mixture 235 µl): _____ µM
- NADPH : weight: _____ mg vol: _____ mL
  concentration in reaction mixture(235 µl): _____ mM

- Sodium phosphate buffer prepared: _____
- Fluorescamine prepared: _____

Results verified by : _____

	Well #	Sample No.	Buffer (µl)	BSA (µl)	Resorufin (µl)	Microsomes (µl)	ER (µl)	NADPH (µl)	Fluorescamine (µl)
Standards	1 A-C		-	-	-		-	-	-
Standards	1 D-F		-	-	-		-	-	-
Standards	2 A-C	BSA and	135	0	0		50	50	100
Standards	2 D-F	RESORUFIN	120	10	5		50	50	100
Standards	3 A-C	standards	105	20	10		50	50	100
Standards	3 D-F		75	40	20		50	50	100
Standards	4 A-C		50	60	25		50	50	100
Standards	4 D-F		15	80	40		50	50	100
CONTROL BLANK	A 5						50	0	100
CONTROL	A 6-8						50	50	100
SAMPLE BLANK	B 5						50	0	100
SAMPLE	B 6-8						50	50	100
SAMPLE BLANK	C 5						50	0	100
SAMPLE	C 6-8						50	50	100
SAMPLE BLANK	D 5						50	0	100
SAMPLE	D 6-8						50	50	100
SAMPLE BLANK	E 5						50	0	100
SAMPLE	E 6-8						50	50	100
SAMPLE BLANK	F 5						50	0	100
SAMPLE	F 6-8						50	50	100

NOTE: total volume in each well must be 335 µL